Maturation of the Hepatitis A Virus Capsid Protein VP1 Is Not Dependent on Processing by the 3CPro Proteinase

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Most details of the processing of the hepatitis A virus (HAV) polyprotein are known. Unique among members of the family Picornaviridae, the primary cleavage of the HAV polyprotein is mediated by 3Cpro, the only protease known to be encoded by the virus, at the 2A/2B junction. All other cleavages of the polyprotein have been considered to be due to 3Cpro, although the precise location and mechanism responsible for the VP1/2A cleavage have been controversial. Here we present data that argue strongly against the involvement of the HAV 3Cpro protease in the maturation of VP1 from its VP1-2A precursor. Using a heterologous expression system based on recombinant vaccinia viruses expressing the full-length or truncated capsid protein precursors, we show that the C terminus of the mature VP1 capsid protein is located near residue 764 of the polyprotein. However, a proteolytically active HAV 3Cpro that was capable of directing both VP0/VP3 and VP3/VP1 cleavages in vaccinia virus-infected cells failed to process the VP1-2A precursor. Using site-directed mutagenesis of an infectious molecular clone of HAV, we modified potential VP1/2A cleavage sites that fit known 3Cpro recognition criteria and found that a substitution that ablates the presumed 3Cpro dipeptide recognition sequence at Glu764-Ser765 abolished neither infectivity nor normal VP1 maturation. Altered electrophoretic mobility of VP1 from a viable mutant virus with an Arg764 substitution indicated that this residue is present in VP1 and that the VP1/2A cleavage occurs downstream of this residue. These data indicate that maturation of the HAV VP1 capsid protein is not dependent on 3Cpro processing and may thus be uniquely dependent on a cellular protease.
TABLE 1. Infectivity of HAV mutant RNA transcripts in FRhK-4 cells

<table>
<thead>
<tr>
<th>Construct aa 745 (nt 2980-82)</th>
<th>aa 764 (nt 3037-39)</th>
<th>aa 776 (nt 3073-75)</th>
<th>aa 791 (nt 3118-20)</th>
<th>Virus titer (RFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Glu (UGG) Glu (GAG) Glu (GAG)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$5 	imes 10^5 - 1 	imes 10^7$</td>
</tr>
<tr>
<td>745N Asn (AAC) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>10^7</td>
</tr>
<tr>
<td>745R Arg (CGG) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>764Q Gin (CAG) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>ND</td>
</tr>
<tr>
<td>764R Arg (CGA) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2 x 10^6</td>
</tr>
<tr>
<td>776Q Gin (CAG) — — —</td>
<td>Arg (CGG) — — —</td>
<td>Glu (GAA) — — —</td>
<td>—</td>
<td>2 x 10^6</td>
</tr>
<tr>
<td>776R Arg (CGG) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>791Q Gin (CAG) Glu (GAG) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$6 	imes 10^5 - 3 	imes 10^5$</td>
</tr>
<tr>
<td>791D Asp (GAC) — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5 x 10^7 - 5 x 10^8</td>
</tr>
<tr>
<td>791R Arg (CGG) Lethal NA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>NA</td>
</tr>
</tbody>
</table>

*wt, wild type (p5’P2P3-18f RNA transcript); —, identical to wild type; NA, not applicable; ND, not done.

After infection with virus rescued from transfection lysates.

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associates with VP0 and VP3 to form pentamers, the first intermediate in the morphogenesis of HAV particles (2, 5). The mature capsid protein VP1 is subsequently derived from the VP1-2A precursor later in the morphogenesis process, although preparations of infectious virus particles often contain detectable quantities of VP2-1A (2, 5). The mechanism by which the 2A moiety is cleaved from the VP1-2A precursor is not known. However, purified recombinant 3Cpro has been shown to cleave relevant HAV substrates that were generated in cell-free translation reactions, suggesting that 3Cpro may be responsible for the VP1/2A cleavage (20, 25). More recently, Probst et al. (24) have presented data suggesting that 3Cpro directs the cleavage between VP1 and 2A at a Glu-Ser dipeptide sequence that is present in most HAV strains at residues 764 and 765 of the polyprotein (Glu-764-Ser; amino acid numbering is from the first AUG). This would result in a VP1 protein of 273 amino acid residues, since the N terminus of the mature capsid protein VP1 has been isolated from virions, microsequenced, and shown to be located at residue 492 (Val) of the polyprotein (12, 19).

Here, however, we present data that argue strongly against the involvement of the HAV 3Cpro protease in the maturation of VP1 from its VP1-2A precursor. We show that the C terminus of the mature capsid protein VP1 is located near but downstream of, residue 764 of the polyprotein. Furthermore, we demonstrate that 3Cpro is incapable of directing the cleavage of VP1 from the VP1-2A precursor in vivo, using recombinant vaccinia viruses that express relevant HAV substrates, and show that a substitution that ablates the presumed 3Cpro dipeptide recognition sequence at positions 764-765 of the polyprotein (Glu764-Ser; amino acid numbering is from the first AUG). This would result in a VP1 protein of 273 amino acid residues, since the N terminus of the mature capsid protein VP1 is located near but downstream of, residue 764 of the polyprotein. Furthermore, we demonstrate that 3Cpro is incapable of directing the cleavage of VP1 from the VP1-2A precursor in vivo, using recombinant vaccinia viruses that express relevant HAV substrates, and show that a substitution that ablates the presumed 3Cpro dipeptide recognition sequence at positions 764-765 of the polyprotein neither abolishes infectivity of the HAV nor eliminates the normal maturation of the VP1 capsid protein. These data strongly refute the hypothesis that the maturation of VP1 is dependent on 3Cpro processing of the VP1-2A precursor and suggest a novel role for an unknown cellular protease in processing of a picornavirus polyprotein.

MATERIALS AND METHODS

Cell cultures and viruses. Fetal rhesus kidney (FRhK-4) cells were used for rescue of infectious HAV following transfection with synthetic, genome-length HAV RNA transcripts (7) and for the in vivo expression of HAV polypeptides following infection with recombinant vaccinia viruses. African green monkey kidney (BS-C-1) cells were used for radioimmunoprecipitation assays (RIFA) (16) to characterize the replication phenotype of mutant HAVs and to determine the titer of virus stocks. These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL or Eurobio) supplemented with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (DMEM-5%) and a mixture of nonessential amino acids (Gibco BRL). Human 143B thymidine kinase-deficient cells were used for the isolation of recombinant vaccinia viruses, and monkey kidney (CV1) or human cervix carcinoma (HeLa) cells were used for propagation of these viruses. Both of these cell lines were maintained in DME-5%.

The wild-type vaccinia virus, Copenhagen strain, and its thermo-sensitive ts derivative (10) were propagated in HeLa cells and purified by centrifugation through a sucrose gradient. Titers of infectious virus were determined in CV1 cells. VP1-7,3, a recombinant vaccinia virus expressing T7 DNA-dependent RNA polymerase (11), was obtained from B. Moss (National Institutes of Health, Bethesda, Md.). Infectious HAV was obtained by transfection of FRhK-4 cells with synthetic RNA derived from a chimeric cDNA (p5’P2P3-18f) (29) containing the P1 segment of a relatively low passage, cell culture-adapted variant of the HM175 strain (6, 7) in the background of a rapidly replicating, cytopathic HM175 variant, 18f (17).

HAV expression plasmids and generation of recombinant vaccinia viruses. For expression of HAV polypeptides in eukaryotic cells, appropriate HAV cDNA fragments were cloned into plasmid pMTM1 downstream of the T7 RNA polymerase promoter (22). Recombinant vaccinia viruses were generated from these plasmids by homologous DNA recombination as described previously (21). Plasmid pTM/P1-2A was constructed by PCR amplification of nucleotides (nt) 748 to 3255 of the HAV sequence from p5’P2P3-18f (29) with a 5’ oligonucleotide primer complementary to nt 748 to 775 and a 3’ primer complementary to nt 3231 to 3255 and possessing an extension designed to create a Spe1 restriction site at the 3’ end of the amplifier. The resulting DNA fragment was digested by Spe1, 5’ phosphorylated, and inserted into the Neol and Spe1 sites of the pTM1 polylinker.

To create pTM1 derivatives expressing a series of truncated HAV capsid protein precursors, nt 2221 to 2982, 2221 to 3039, 2221 to 3075, or 2221 to 3120 were PCR amplified from p5’P2P3-18f cDNA, using a series of 3’ primers containing a 3’ Spe1 site and a 5’ primer complementary to nt 2221 to 2226. The resulting PCR amplimers were digested with Neol (nt 2827) and Spe1 and inserted in lieu of the corresponding full-length fragment (nt 2827 to 3255) of pTM/P1-2A. These plasmids encode truncated capsid protein precursors, with C termini corresponding to residues 745 (p745P1-2A), 764 (p764P1-2A), 776 (p776P1-2A), and 791 (p791P1-2A) of the polyprotein, or potential VP1 proteins of 254, 273, 265, or 300 residues, respectively.

Plasmid pTM/2BC-P3 was constructed from a dicistronic cDNA with an encephalomyocarditis virus internal ribosomal entry site insertion between 2A and 2B sequences of 5’P2P3-18f (pHAV-2AE2B) (2a). The Apa718-BamHI restriction fragment, which included the 132 3’-terminal nucleotides of the encephalomyocarditis internal ribosomal entry site, the initiation codon, HAV 2BC and P3 sequences, as well as HAV 3’-terminating region and poly(A) sequence, was sequenced from the junction of the polyprotein sequence with the C terminus of the polyprotein, or potential VP1 proteins of 254, 273, 265, or 300 residues, respectively.

Construction of mutated full-length HAV cDNAs by site-directed mutagenesis. Potential 3Cpro cleavage sites which may represent the VP1/2A junction were altered in the infectious molecular clone, p5’P2P3-18f (29), using three different strategies for site-directed mutagenesis. For mutants 764Q, 764R, 776Q, 776R, and 791R (Table 1), the isolated SacI-EcoRI fragment (nt 3002 to 4990) of p5’P2P3-18f was inserted into the polylinker of phage M13mp19 DNA and subjected to site-directed mutagenesis according to the method of Taylor et al. (28). For mutants 745N and 745R (Table 1), the BstEII-HindIII segment (nt 2037 to 3002) of p5’P2P3-18f was PCR amplified by using a 3’ oligonucleotide primer with nucleotide substitutions in the relevant codon. For mutants 791D and 791Q (Table 1), p5’P2P3-18f was used as a template for PCR-mediated mutagenesis by an adaptation of the method described by Stemmer and Morris (27). All mutated cDNA segments were sequenced to exclude spurious mutations prior to their reintroduction into the background of p5’P2P3-18f.
3Cpro precursor in this expression system. To generate mature 3Cpro proteinase as a major product (Fig. 1), transfected cells were maintained at 37°C for 7 days before processing. HAV polypeptide expression assays. Segments of the HAV polyprotein were expressed in FRhK-4 cells (2 x 10^6 cells in 35-mm-diameter petri dishes) by coinfection of the cells with VTF7-3 and vaccinia virus-HAV recombinants expressing full-length or truncated capsid protein precursor (vv-P1-2A) and/or a 3Cpro precursor (vv-2BC-P3), each at a multiplicity of infection (MOI) of 5 PFU per cell.

Stocks of viable HAV mutants were used to infect FRhK-4 cells (2 x 10^6 cells in 35-mm-diameter petri dishes) at an MOI of 1 to 2 radioimmunofocus-forming units (RFU) per cell (16).

Immunoblot detection of HAV proteins. Cytoplasmic extracts were prepared at 20 h postinfection (p.i.) for vaccinia virus infections or at 24, 48, 72, or 96 h p.i. (for HAV infections) by lysis of cells in 200 μl of 50 mM Tris-Cl (pH 7.5)-150 mM NaCl-1 mM EDTA-1% Nonidet P-40-0.1% sodium deoxycholate containing 25 μg of aprotinin per ml. A 10- to 20-μl aliquot was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer onto a polyvinylidene difluoride membrane (Amersham). Non-specific binding sites were blocked in phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 5% nonfat milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with a mixture of HAV anti-VP1 and anti-VP2 guinea pig antibodies, diluted 1:4,000 and 1:8,000, respectively, in PBST containing 1% bovine serum albumin (PBST-BSA). After four washes with PBST, the membrane was incubated with anti-guinea pig antibodies conjugated to horseradish peroxidase (Sigma) diluted in PBST-BSA for 1 h at room temperature. After four washes with PBST, the HAV polypeptides were visualized by chemiluminescence (ECL Plus; Amersham).

RESULTS

Processing of the HAV P1-2A polypeptide by 3Cpro proteinase expressed by recombinant vaccinia viruses. To study proteolytic cleavage of the HAV polyprotein by the 3Cpro proteinase of HAV, we expressed segments of the polyprotein in FRhK-4 cells in a hybrid T7-vaccinia expression system (11, 22). HAV cDNA sequences were cloned into plasmid pTM1 under control of the T7 promoter and recombinant vaccinia virus P2P3-18f virus (29) as well as their positions within the polyprotein are indicated in these cell lysates, indicating that the proteolytically active 3Cpro proteinase was not able to direct cleavage at the VP1/2A junction in this system. Mature VP1 capsid protein from HAV-infected cells comigrates with a recombinant VP1 molecule of 273 amino acid residues (VP1273). In an effort to determine the approximate location of the C terminus of the mature VP1 capsid protein, to determine whether the capsid protein precursor, P1-2A, could be cleaved in trans by 3Cpro in this expression system, FRhK-4 cells were infected with VTF7-3 and vv-P1-2A, with or without coinfection with vv-2BC-P3, which expresses the 3Cpro precursor. Cell lysates were subjected to SDS-PAGE, and HAV polypeptides visualized in immunoblots with a mixture of anti-VP1 and anti-VP2 antibodies. The 94-kDa P1-2A precursor was processed only in cells expressing the 2BC-P3 protease precursor, yielding VP1-2A (PX) and VP0 (Fig. 2B). This result demonstrates that 3Cpro expressed from vv-2BC-P3 is able to process P1-2A in trans at both the VP0/VP3 and VP3/VP1 junctions. However, no mature VP1 was identified in these cell lysates, indicating that the proteolytically active 3Cpro proteinase was not able to direct cleavage at the VP1/2A junction in this system.

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we constructed a series of recombinant vaccinia viruses expressing truncated P1-2A capsid protein precursors: aa 1 to 745 (vv-P1745), 1 to 764 (vv-P1764), 1 to 776 (vv-P1776), and 1 to 791 (vv-P1791). The C-terminal residues of these truncated precursors correspond to each of the four potential 3Cpro cleavage sites that may represent the VP1/2A junction: Gln745-Ser, Glu764-Ser, Glu776-Ser, and Glu791-Ser (Fig. 1). These sites were identified on the basis of the substrate recognition criteria of picornaviral 3Cpro proteinases, which include a requirement for a Gln or possibly a Glu residue at the P1 position immediately preceding the scissile peptide bond (3). We examined the processing of these truncated P1-2A substrates by 3Cpro expressed in cells that were coinfected with vv-2BC-P3 and vTF7-3 (Fig. 3A). Products containing VP1 residues were identified by immunoblotting with anti-VP1 antibodies. As expected, the proteolytic cleavage of these substrates by 3Cpro produced C-terminally truncated VP1-2A polypeptides representing aa 492 to 745, 492 to 764, 492 to 776, and 492 to 791 (Fig. 3A).

When subjected to SDS-PAGE, the electrophoretic mobilities of these truncated VP1-2A molecules bracketed that of mature VP1 isolated from HAV-infected cells (Fig. 3A, lanes 2 to 4). The natural VP1 protein (Fig. 3A, lane 3) roughly comigrated with the recombinant VP1764 product derived from the vv-P1764 precursor (lane 4) that contains 273 amino acid residues. To determine whether we could detect subtle differences in the electrophoretic mobilities of these two proteins, equal amounts of cytoplasmic extracts from HAV-infected cells and cells coinfected with vTF7-3, vv-P1764, and vv-2BC-P3 were loaded onto an SDS-polyacrylamide gel between lanes containing the individual extracts. As shown in Fig. 3B (lane 3+4), the mixture of the natural VP1 and recombinant VP1764 proteins migrated as a single band. This result suggests that the C terminus of the mature VP1 capsid protein lies close to, if not at, residue 764.

Similar to the results we obtained for cells expressing the entire P1-2A polyprotein segment as a substrate for 3Cpro (Fig. 2), neither of the two recombinant truncated P1-2A precursor proteins that contain the N terminus of 2A (P1776 and P1791) were completely processed into mature VP1 in the presence of the protease (Fig. 3A).

Infectivity of mutated HAV RNA transcripts. To ascertain whether any of the four potential VP1/2A cleavage sites listed above constitute actual substrates for the 3Cpro protease within the context of the full-length HAV polyprotein, substitutions were introduced at the P1 residue of these sites within a genome-length, infectious HAV cDNA clone, p59P2P3-18f. The P1 residue represents the most important determinant of 3Cpro specificity. Two types of mutations were engineered at each of these putative junctions (Table 1). One set of mutations was designed to preserve or even enhance 3Cpro recognition and cleavage (Gln745→Asn and Glu764,776,791→Gln), while a second set of substitutions were nonconservative and designed to abolish 3Cpro recognition of the dipeptide sequence (Gln745 and Glu764,776,791→Arg). In addition, we introduced a conservative substitution that maintained the charge of the Glu residue at position 791 (Glu791→Asp). For each mutation, we attempted to rescue infectious HAV from two independent clones by in vitro transcription of the cDNA with SP6 RNA polymerase, followed by liposome-mediated transfection of the RNA transcripts into permissive FRhK-4 cells (see Materials and Methods). Cell lysates were prepared 2 weeks after transfection, and the presence of virus was determined by RIFA in BS-C-1 cells.

No infectious virus was recovered from any of the mutants with substitutions involving the potential Gln745-Ser or Glu776
Ser dipeptide cleavage sites: 745N, 745R, 776Q, or 776R (Table 1). The lethal nature of these mutations, even when the substitution was conservative in terms of the likelihood of preserving 3Cpro recognition (Table 1), suggests that these dipeptide sequences are unlikely to function as substrates for 3Cpro cleavage during virus replication. Alternatively, if these are sites of 3Cpro cleavage, they must possess highly stringent requirements for 3Cpro recognition.

No virus was rescued from the 791R mutant (Table 1). The lethal nature of this mutant is consistent with what would be expected if Glu791-Ser were a 3Cpro cleavage site. In contrast, an HAV-specific cytopathic effect was observed after transfection with the 791D RNA transcripts, and virus was detected in lysates of these cells by RIFA (Fig. 4). No cytopathic effect was observed following transfection with the 791Q mutant, but small viral replication foci were detected by RIFA in BS-C-1 cells infected with lysates of these cells (Table 1 and Fig. 4). With both the 791Q and 791D mutants, the titers of infectious HAV were lower and RIFA foci were smaller than those of the parental virus (Table 1). Moreover, they produced RIFA foci only slightly smaller than (764R) or the same size as (764Q) those of the parental virus (Fig. 4). The latter observation is particularly important, since it indicates that the Glu→Arg substitution in 764R, which would be expected to abolish 3Cpro recognition of the Glu764-Ser dipeptide, had no appreciable effect on replication of the virus.

The viable HAV mutants that were rescued from transfected FRhK-4 cells were subjected to an additional passage in these cells. This led to an amplification of the titer of the 791Q mutant, which nonetheless remained 10- to 100-fold lower than that of parental virus (Table 1). For each of the mutants, however, the size of the RIFA foci remained unchanged after this additional passage (data not shown). In all cases, the RNA sequences of the viruses recovered, either directly from the transfected cells or following an additional passage in FRhK-4 cells, retained the nucleotide substitutions that had been introduced at the suspect 3Cpro cleavage sites by site-directed mutagenesis.

Cleavage at the VP1/2A junction during replication of virus with amino acid substitutions at Glu764 or Glu791. Since the VP1/2A precursor is present in small quantities in some purified preparations of infectious HAV particles (2, 5), the VP1/2A cleavage might not be necessary for replication of the virus. If this were the case, then the viable mutant viruses with substitutions at Glu764 or Glu791 could still have a defect in their ability to fully process the VP1-2A precursor to mature VP1. To exclude this possibility, we infected FRhK-4 cells with these mutants and at various times p.i. prepared cytoplasmic extracts for immunoblot analysis (Fig. 5). VP1- and VP2-reactive proteins were detected with a mixture of anti-VP1 and anti-VP2 antibodies. VP0 and/or VP2 were detected in lysates of cells infected with each of these mutants with kinetics similar to those of the parental virus (Fig. 5).

Each of the VP1-containing polypeptides (P1-2A, VP1-2A, and the mature VP1) could be detected within 48 h of infection with the 791Q and 791D mutants at an MOI of 1 RFU/cell (Fig. 5A). Between 48 and 72 h p.i., the P1-2A precursor appeared to be progressively converted to VP1-2A and then fully processed into VP1 in cells infected with either mutant (Fig. 5A). The processing of the 791Q mutant may have been somewhat delayed, as a significant amount of the P1-2A product remained present as late as 72 h p.i. However, P1-2A and VP1-2A were almost completely processed into VP1 in cells infected with each of these mutant viruses by 96 h p.i. (Fig. 5A). Interestingly, the electrophoretic mobilities of the P1-2A and VP1-2A products were slightly altered by the Glu791→Gln change introduced at residue 791 of the 791Q mutant compared to those of parental virus. However, the electrophoretic mobilities of the fully processed VP1 proteins were identical for both mutants and parental virus (Fig. 5A). This result indicates that the C terminus of VP1 lies upstream of residue 791.

The fully processed VP1 protein was also produced in FRHk-4 cells infected with the 764Q and 764R mutants at an MOI of 2 RFU/cell (Fig. 5B). There were no apparent delays in the processing of the VP1 intermediates in comparison to the wild-type virus. Importantly, however, the electrophoretic mobilities of the P1-2A, VP1-2A, and VP1 proteins were each significantly altered by the Glu→Gln substitution at residue 764 of the mutant 764Q and even more by the Glu→Arg change in 764R (Fig. 5B). This finding indicates that the mature VP1 protein contains residue 764 and that the VP1/2A cleavage must therefore be downstream of the potential 3Cpro cleavage site at Glu764/Ser.
Viruses of the genus *Hepatovirus* are unique among the *Picornaviridae* with respect to the primary cleavage of the viral polyprotein, which is carried out by the viral 3C<sup>pro</sup> proteinase at the 2A/2B junction (13, 21). The capsid protein precursor, polypeptide P1-2A, is subsequently cleaved by 3C<sup>pro</sup> to generate VP0, VP3, and VP1-2A (Fig. 2 and references 20, 24, and 26). In contrast to these well-documented events, considerable uncertainty has surrounded the identification of the junction between VP1 and 2A and the mechanism of this cleavage. One difficulty has been that we and others (24) have been unable to detect the 2A protein in HAV-infected cells. This has made it impossible to purify this protein and determine its N-terminal residue by microsequencing. Our inability to detect 2A could be because it is highly unstable and rapidly degraded, and/or because it is not released from VP1-2A as an intact protein. The role of the 2A polypeptide sequence in virus replication has also been elusive. The 2A proteins of hepatoviruses show no sequence homologies with the 2A proteinase of the enteroviruses and rhinoviruses nor with the cardiogirus 2A protein. The HAV 2A polypeptide is present as a C-terminal extension of VP1 (VP1-2A = PX) in viral pentamers but is generally absent or present in only small quantities in mature virions (2, 5). The mechanism of the VP1/2A cleavage remains unclear, as indicated by the lack of unambiguous reports concerning 3C<sup>pro</sup>-mediated cleavage of VP1 from various substrates (13, 20, 24–26).

Several approaches were undertaken in this study in an effort to elucidate the process leading to maturation of the VP1 capsid protein. We determined the approximate C terminus of the mature VP1 protein that is present in infectious virions, by comparing its electrophoretic mobility in SDS-PAGE with the mature VP1 protein isolated from HAV virions to have an electrophoretic mobility indistinguishable from that of a recombinant VP1 protein with a C terminus at Glu<sup>764</sup> (Fig. 3B). This result demonstrated that the VP1 C terminus is located close to, if not at, residue 764 of the polyprotein.

Crystallographic studies of the HAV 3C<sup>pro</sup> proteinase suggest that a Glu-Ser dipeptide, such as that present at residues 764-765 of the HAV polyprotein, could constitute a substrate for this proteinase, even though 3C<sup>pro</sup> has been shown to exhibit a marked preference for Glu at the P<sub>1</sub> position immediately upstream of the scissile bond (1, 3, 14). In support of this argument, the predicted HAV 3A/3B cleavage site, which has not yet been confirmed by protein sequencing, is also thought to involve a Glu residue at the P<sub>1</sub> position (in this case, a Glu-Gly dipeptide). However, although we have recently shown that a Glu-Ser dipeptide is partially cleaved in trans by 3C<sup>pro</sup> when introduced in lieu of the normal Gln-Ala dipeptide at the 2A/2B site, we have found that a Leu<sup>1-6</sup>-Pro<sup>3</sup>-Thr<sup>3</sup>-Glu<sup>1</sup>-Ser<sup>1</sup>-pentapeptide sequence (which corresponds to the sequence context of the putative 3C<sup>pro</sup> cleavage of VP1/2A Glu<sup>764</sup>-Ser<sup>1</sup>) is not cleaved at all when introduced at the 2A/2B junction (our unpublished results). This is probably due to the additive deleterious effects of two unfavorable residues,
the Pro at the P₁ position as well as the Glu at the P₁ position (2b). Furthermore, a Val residue, such as found at position 764 of the attenuated HM175 p35 strain (6), is not a suitable P₁ residue for 3C⁰⁰⁰ trans cleavage (our unpublished results). All of these data suggest that 3C⁰⁰⁰-mediated cleavage of the HAV polyprotein at the Glu⁷⁶⁴-Ser⁷⁶⁵ dipeptide is not a very likely scenario, despite the fact that Glu⁷⁶⁴ is close to or at the C terminus of VP1 (Fig. 3B). That 3C⁰⁰⁰ is likely not to cleave this dipeptide is further indicated by the failure of the HAV protease to process VP1-2A into a mature VP1 protein when P₁-2A and 2BC-P₃ polypeptides were expressed in vivo by recombinant vaccinia viruses, whereas 3C⁰⁰⁰ is fully active to process P₁-2A at other junctions (Fig. 2).

These observations led us to test whether the Glu⁷⁶⁴-Ser dipeptide was an actual substrate for 3C⁰⁰⁰ in the context of the full-length polyprotein by introducing various amino acid substitutions at Glu⁷⁶⁴ that were designed to either maintain or abolish potential recognition by the 3C⁰⁰⁰ protease. The transfection of FRhK-4 cells with an HAV RNA transcript encoding a Glu⁷⁶⁴→Arg substitution at this putative P₁ residue resulted in the rescue of a mutant virus (764R) with replication properties similar to those of the parental virus (Table 1 and Fig. 4). This substitution should completely abolish 3C⁰⁰⁰ sub-

Fig. 4). This substitution proved lethal to replication, we found that conservation of the charge of the putative P₁ residue (Glu⁷⁶⁴→Asp substitution) was more important than maintaining a 3C⁰⁰⁰ recognition sequence (Glu⁷⁶⁴→Gln substitution) to preserve virus replication. Thus, it seems that there is no sequence downstream of residue 764 in the vicinity of the VP1-2A cleavage that is recognized by 3C⁰⁰⁰. This finding suggests that the cleavage of VP1 from its VP1-2A precursor is not dependent on 3C⁰⁰⁰ which is the only protease known to be expressed by HAV.

This observation raises the possibility that a cellular proteinase contributes to the mechanism of VP1/2A cleavage during the replication of HAV. If so, such a major role for a cellular proteinase in the processing of the polyprotein would be unique to HAV among all picornaviruses. This interpretation leaves unanswered the question as to why no maturation of the VP1 protein from its VP1-2A precursor was observed in FRHK-4 cells expressing P₁-2A and 2BC-P₃ (3C⁰⁰⁰) from recombinant vaccinia viruses (Fig. 2). The entire HAV protein complement is present in this system, and FRHK-4 cells are permissive for virus replication and thus must express any putative cellular proteinase involved in maturation of the HAV capsid protein. Perhaps such a proteinase is induced by HAV infection and not present in sufficient quantities in uninfected FRHK-4 cells to render its detection possible. A difference in the abundance of this unknown cellular proteinase in the different cell types used in our study and in that of Probst et al. (24) may explain the discrepancy relating to whether processing of a P₁-2A substrate to a mature VP1 product can occur. Alternatively, this unknown proteinase may be sequestered within an isolated compartment of the FRHK-4 cells which is not accessible to vaccinia-expressed HAV polypeptides. However, it cannot be ruled out that VP1-2A becomes a competent substrate for this proteinase only after assembly of the virus particle.

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REFERENCES